Supporting Information

Rardin et al. 10.1073/pnas.1302961110

SI Methods and Materials

Materials. HPLC solvents including acetonitrile and water were obtained from Burdick & Jackson. Reagents for protein chemistry including iodoacetamide, DTT, ammonium bicarbonate, formic acid, trifluoroacetic acid, trichostatin A, dodecyl-maltoside, urea, nicotinamide, and BSA were purchased from Sigma Aldrich. Acetylated synthetic peptides containing stable isotope labeled lysine or arginine residues ($^{13}C_6{}^{15}N_2$-Lys and $^{13}C_6{}^{15}N_4$-Arg, respectively) were obtained from Thermo Fisher Scientific at >95% purity. Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Thermo, and HLB Oasis SPE cartridges were purchased from Waters. Protein G agarose was obtained from Pierce, and proteomics grade trypsin was from Promega. Anti–glutamate dehydrogenase antibody was purchased from Rockland. Trypsin-predigested β-galactosidase (a quality control standard) was purchased from AB SCIEX.

MS and Chromatographic Parameters. All samples used for MS1 Filtering experiments were analyzed by reverse-phase liquid chromatography-electrospray ionization-MS/MS using an Eksigent Ultra Plus nano-LC 2D HPLC system connected to a quadrupole time-of-flight TripleTOF 5600 mass spectrometer (AB SCIEX) in direct injection mode. The autosampler was operated in full injection mode overfilling a 1-μL loop with 3 μL analyte for optimal sample delivery reproducibility. Briefly, after injection, peptide mixtures were transferred onto the analytical C18-nano-capillary LC column (C18 Acclaim PepMap100, 75-μm ID × 15 cm, 3-μm particle size, 100-Å pore size; Dionex) and eluted at a flow rate of 300 nL/min using the following gradient: at 3% (vol/vol) solvent B in A (from 0 to 13 min), 3–7% (vol/vol) solvent B in A (from 13 to 16 min), 7–25% (vol/vol) solvent B in A (from 16 to 48 min), 25–40% (vol/vol) solvent B in A (from 48 to 65 min), 40–90% (vol/vol) solvent B in A (from 65 to 75 min), and at 90% (vol/vol) solvent B in A (from 75 to 85 min), with a total run time of 120 min including mobile phase equilibration. Solvents were prepared as follows: mobile phase A, 2% acetonitrile/98% of 0.1% formic acid (vol/vol) in water; mobile phase B, 98% acetonitrile/2% of 0.1% formic acid (vol/vol) in water. Mass spectra and tandem mass spectra were recorded in positive-ion and high-sensitivity mode with a resolution of ∼35,000 full-width half-maximum in MS1 and 15,000 in MS2. The nanospray needle voltage was typically 2,400 V in HPLC-MS mode. After acquisition of approximately five to six samples, TOF MS spectra and TOF MS/MS spectra were automatically calibrated during dynamic LC-MS and MS/MS autocalibration acquisitions injecting 25 fmol β-galactosidase. For collision-induced dissociation tandem MS (CID-MS/MS), the mass window for precursor ion selection of the quadrupole mass analyzer was set to ±1 *m/z*. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. Advanced information-dependent acquisition (IDA) was used for MS/MS collection on the TripleTOF 5600 to obtain MS/MS spectra for the 20 most abundant parent ions following each survey MS1 scan (allowing typically for 50 ms acquisition time per each MS/MS, which for some experiments was increased to 75 ms). Dynamic exclusion features were based on value M not *m/z* and were set to an exclusion mass width of 50 mDa and an exclusion duration of 15–20 s. MS/MS spectra for all identified peptides may be viewed using Panorama (1) at the following location: https://skyline.gs.washington.edu:9443/labkey/project/Gibson/Gibson_Reviewer/begin.view?; raw data files are available for download at the following location: ftp://sftp.buckinstitute.org/Gibson.

For selected reaction monitoring (SRM), samples were analyzed by nano-LC-SRM/MS on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX). Chromatography was performed on a NanoLC-Ultra 2D LC system (Eksigent) with buffer A [0.1% (vol/vol) formic acid] and buffer B (90% acetonitrile in 0.1% formic acid). Digests were separated on a 15-cm-long, 75-μm reversed phase C18 column (3 μm, 120Å; Eksigent) in a cHiPLC nanoflex chip configuration at a flow rate 300 nL/min. Gradient was 3% B from 0 to 5 min, increased to 15% B over 3 min, and increased to 35% B over the next 34 min. Peptides were ionized using a PicoTip emitter (20 μm, 10-μm tip; New Objective). Data acquisition was performed using Analyst 1.5.1 (AB SCIEX) with an ion spray voltage of 2,300 V, curtain gas of 20 psi, nebulizer gas of 15 psi, and interface heater temperature of 150 °C.

The transitions, dwell times, and collision energy are listed in Dataset S6. Four transitions were assayed per peptide. The declustering potential and collision energy for each transitions were optimized with using stable isotope-labeled synthetic peptides, labeled at the C terminus with either $^{13}C_6{}^{15}N_2$-Lys or $^{13}C_6{}^{15}N_4$-Arg. Retention time scheduling was also used with a retention time window of 240 s and a target scan time of 1.5 s. A value of 40 was used as the collision cell exit potential for all transitions. Selected reaction monitoring (SRM) transitions were acquired at unit resolution both in the first and third quadrupoles (Q1 and Q3). Standard curves were performed in triplicate by spiking in the stable isotope-labeled peptide to determine the linear range, limit of detection (LOD), and limit of quantitation (LOQ) in a background matrix of 250 ng of protein (2). Skyline postacquisition software was used to process all SRM data (3). Samples were analyzed in duplicate with 25 fmol of each heavy peptide spiked in. Each transition was individually integrated to generate peak areas, and the peak area of the most intense area was used for analysis.

Bioinformatic Database Searches. Mass spectral data sets were analyzed and searched using Mascot server version 2.3.02 (Matrix Sciences) and ProteinPilot (revision 148085; AB SCIEX 4.0) using the Paragon algorithm (4.0.0.0, 148083). All data files were searched using the SwissProt 2011_08 database with a total of 531,473 sequences but were restricted to *Mus musculus* (16,441 protein sequences). Search parameters in Mascot for acetylated peptides were as follows: trypsin digestion with four missed cleavages to account for the inability of trypsin to cleave at acetylated lysine residues. Trypsin specificity was set to C-terminal cleavage at lysine and arginine. Variable modifications included lysine acetylation, methionine oxidation, conversion of glutamine to pyroglutamic acid, and deamidation of asparagine. Carbamidomethyl cysteine was set as a fixed modification. Precursor ion and fragment ion mass tolerances were set to 20 ppm and 0.2 Da, respectively. Peptides with an expectation value <1% false discovery rate (FDR) were chosen for further data processing.

The following sample parameters were used in Protein Pilot: trypsin digestion, cysteine alkylation set to iodoacetamide, urea denaturation, and acetylation emphasis. Processing parameters were set to "Biological modification," and a thorough ID search effort was used. A local FDR of 1% was chosen using the Protein Pilot FDR analysis tool (PSPEP) algorithm (4). All mass spectral details for acetylated peptides are available in Dataset S1. For non-acetylated peptide searches, the acetylation emphasis was not used, and a peptide confidence value of 95 was chosen; mass spectral details for nonenriched peptides are available in Dataset S5.

Skyline MS1 Filtering Tool Algorithm and Data Analysis. Skyline is an open source software project and can be freely installed. Additional details and tutorials for creating spectral libraries and MS1 Filtering can be viewed on the Skyline website (http://proteome.gs.washington.edu/software/skyline). Spectral libraries were generated in Skyline using the BiblioSpec algorithm (5) from database searches of the raw data files as previously described (6). Raw files were directly imported into Skyline in their native file format, which Skyline achieves using the ProteoWizard data access library (7). After data import, graphical displays of chromatographic traces for the top three isotopic peaks were manually inspected for proper peak picking of MS1 filtered peptides. All quantitations performed in this study were done on the peptide level, using a peptide centric approach. Only the most abundant isotope for each peptide was used for quantitation. Following data extraction, peptide areas were normalized to the spiked in acetyllysine peptide standard (m/z 626.8604^{++}; LVSSVSDLPacKR, where acetylated lysine (acK) is *N*-acetyllysine and R = $^{13}C_6{}^{15}N_4$-Arg) and then multiplied by a normalization factor 1e^7 to ensure all values were >1. The normalized peptide area was then averaged across all WT or SIRT3$^{-/-}$ acquisitions, and a ratio was generated (KO:WT). *P* values were calculated using a two-tailed, unpaired Student *t* test. All details for peptide quantitation using MS1 Filtering are provided in Datasets S3, S4, and S5. The FDR for the SIRT3 target proteins was calculated using the Benjamini–Hochberg procedure.

Conservation Index of Lysine Acetylation Sites. For each mouse protein containing one or more acetylation sites, the full sequence was downloaded from UniProt and then aligned to the nr database by blastpgp (BLAST suite 2.2.18) (8). To construct a high-quality multiple sequence alignment, we applied two criteria to all hits in each alignment sequentially: (*i*) sequence identity between 30% and 94% to the quest mouse protein; and (*ii*) >10 such hits could be found. We then generated a multiple sequence alignment on selected hits and original mouse protein by CLUSTALW (2.0.12) using default parameter settings (9). Finally, we used AL2CO on the output of CLUSTALW to compute conservation index on each aligned column and extracted corresponding values for all annotated acetylation sites (10).

Sample Preparation for Mouse Liver Mitochondrial Protein Lysate. Livers were extracted from five WT and five SIRT3$^{-/-}$ male mice at 17 wk of age following a 24-h fast in the presence of deacetylase inhibitors (10 mM nicotinamide and 0.5 μM trichostatin A). Mitochondria were isolated by differential centrifugation as described previously (11). Mitochondrial protein (1.05 mg/mouse) was denatured with 1% dodecyl-maltoside and 10 M urea per process replicate. Samples were then diluted 1:10, reduced with 4.5 mM TCEP (37 °C for 1 h), alkylated with 10 mM iodoacetamide (30 min at room temperature in the dark), and incubated overnight at 37 °C with sequencing grade trypsin added at a 1:50 enzyme:substrate ratio (wt/wt). Samples were then acidified with formic acid and desalted using HLB Oasis SPE cartridges. Samples were eluted, concentrated to near dryness by vacuum centrifugation, and resuspended in NET buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). Protein digest from each sample (50 μg) was desalted using C-18 zip-tips for total peptide analysis of samples by MS1 Filtering, whereas the remaining 1 mg was used for affinity purification of lysine-acetylated peptides.

Affinity Purification of Lysine-Acetylated Peptides. The polyclonal anti-acetyllysine antibodies (Cell Signaling 9441) were immobilized on protein G agarose beads (4 °C, 2 h) and combined with an equal amount of prewashed anti-acetyllysine agarose antibody conjugate (ImmuneChem ICP0380-100). Acetyllysine peptide standard (100 fmol; m/z 626.8604^{++}; LVSSVSDLPacKR) was added to the digested peptides from the above mitochondrial protein lysate and incubated overnight (4 °C) at a 1:25 antibody:peptide ratio (wt/wt). Beads were washed three times in NET buffer, and the peptides were eluted by washing three times in 1% trifluoroacetic acid/40% acetonitrile (vol/vol). Peptides are concentrated to near dryness by vacuum centrifugation and resuspended in equal amounts of 0.1% formic acid/1% acetonitrile. The acetyllysine peptide enrichments were subsequently desalted using C-18 zip-tips. After evaporation of organic solvents, samples were suspended in 0.1% formic acid/1% acetonitrile and analyzed by LC-MS/MS on the TripleTOF 5600. A solution-only "blank" was run in between sample acquisition to prevent carryover that would affect downstream quantitative analysis.

1. Sharma V, et al. (2012) Panorama: A private repository of targeted proteomics assays for Skyline. *Proceedings of the 60th ASMS Conference on Mass Spectrometry and Allied Topics*, American Society for Mass Spectrometry, May 20th-24th Vancouver, BC (American Society for Mass Spectrometry, Santa Fe, NM).
2. Shrivastava A, Gupta VB (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chron Young Sci* 2(1): 21–25.
3. MacLean B, et al. (2010) Skyline: An open source document. for creating and analyzing targeted proteomics experiments. *Bioinformatics* 26(7):966–968.
4. Shilov IV, et al. (2007) The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol Cell Proteomics* 6(9):1638–1655.
5. Frewen B, MacCoss MJ (2007) Using BiblioSpec for creating and searching tandem MS peptide libraries. *Curr Protoc Bioinform*, pp 20:13.7.1-13.7.12.
6. Schilling B, et al. (2012) Platform-independent and label-free quantitation of proteomic data using MS1 extracted ion chromatograms in skyline: Application to protein acetylation and phosphorylation. *Mol Cell Proteomics* 11(5):202–214.
7. Kessner D, Chambers M, Burke R, Agus D, Mallick P (2008) ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics* 24(21):2534–2536.
8. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402.
9. Larkin MA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21): 2947–2948.
10. Pei J, Grishin NV (2001) AL2CO: calculation of positional conservation in a protein sequence alignment. *Bioinformatics* 17(8):700–712.
11. Rardin MJ, Wiley SE, Naviaux RK, Murphy AN, Dixon JE (2009) Monitoring phosphorylation of the pyruvate dehydrogenase complex. *Anal Biochem* 389(2):157–164.

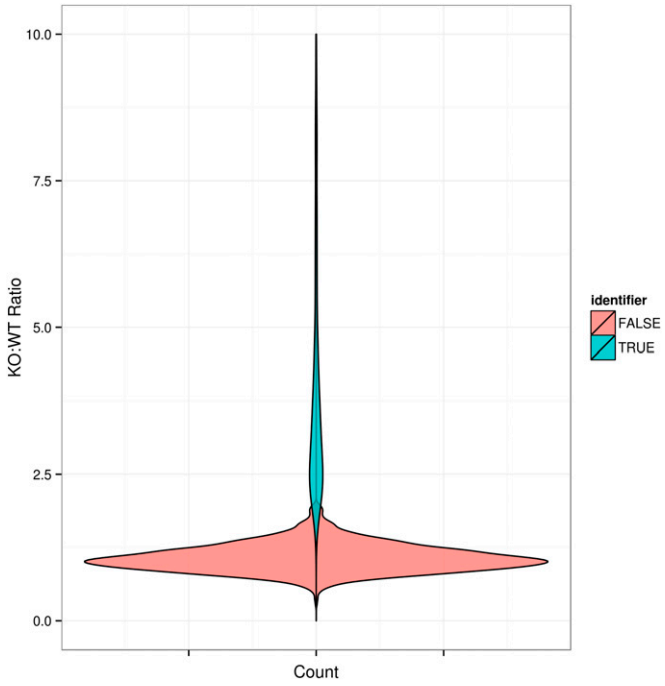

Fig. S1. Volcano plot showing the distribution of acetylation sites quantitated with the majority of sites having a ratio (KO:WT) near 1. Peptides with greater than a twofold change and $P < 0.01$ were considered "true" SIRT3 targets. Two-tailed Student *t* test (*$P < 0.05$, **$P < 0.01$); $n = 5$ for WT and KO with two injection replicates per sample.

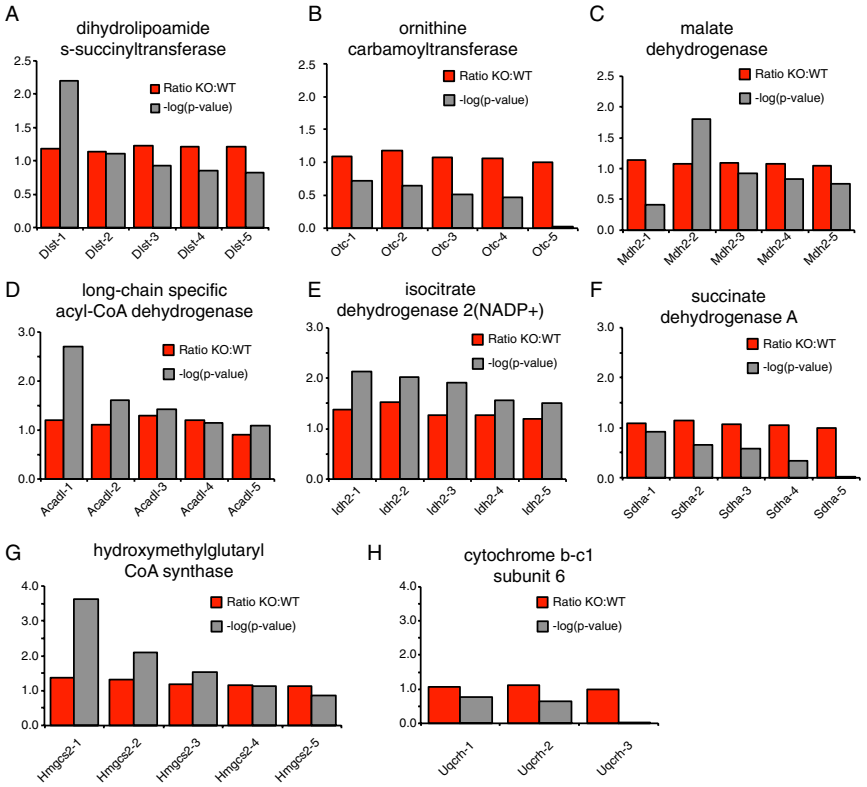

Fig. S2. Quantitation of peptides from mitochondrial digests for determining protein expression levels. Relative ratio (KO:WT) shown in red bars of five separate peptides quantitated by MS1 Filtering from (*A*) dihydrolipoamide s-succinyltransferase (DLST), (*B*) ornithine carbamoyltransferase (OTC), (*C*) malate dehydrogenase (MDH2), (*D*) long-chain specific acyl-CoA dehydrogenase (ACADL), (*E*) isocitrate dehydgrogenase ($NADP^+$) (IDH2), (*F*) succinate dehydrogenase subunit A (SDHA), (*G*) hydroxmethylglutaryl-CoA synthase (HMGCS2), and (*H*) cytochrome *b*-c1 subunit 6 (UGCRH). Two-tailed Student *t* test [−log(*P* value)] shown in gray bars; $n = 5$ for WT and $SIRT3^{-/-}$(KO) with two injection replicates per sample.

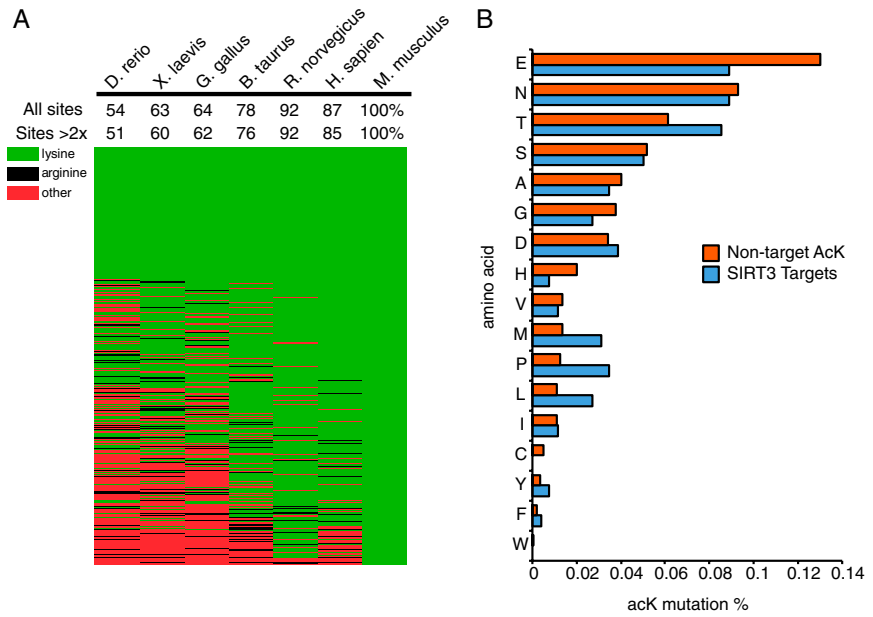

Fig. S3. Conservation of non-SIRT3 regulated acetylation sites. (*A*) Conservation index of acK sites identified in mouse across seven species that were not significantly increased in SIRT3$^{-/-}$ mice. Lysine residues are in green, arginine, which is the most similar amino acid in charge and structure to lysine, is in black, and all other amino acids are in red. (*B*) The percentage of acK sites identified in mouse that are mutated to other amino acids. Sites are distinguished based on whether they were up-regulated in SIRT3$^{-/-}$ animals.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)
Dataset S5 (XLSX)
Dataset S6 (XLSX)